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Evaluation and Clinical Validation of an Alcohol-Based Transport Medium for Preservation and Inactivation of Respiratory Viruses[▽]

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The clinical and public health importance of influenza and other respiratory viruses has accelerated the development of highly sensitive molecular diagnostics, but data are limited regarding preanalytical stages of diagnostic testing. We evaluated CyMol, an alcohol-based transport medium, for its ability to maintain specimen integrity for up to 21 days of storage at various temperatures; for its ability to inactivate virus; and for its compatibility with antigen- or nucleic acid-based diagnostics for respiratory viruses in clinical samples. In mock-infected samples, both universal transport medium (UTM-RT) and CyMol maintained equivalent viral quantities for at least 14 days at room temperature or colder, whereas a dry swab collection maintained viral quantities only if refrigerated or frozen. CyMol inactivated influenza virus within 5 min of sample immersion. UTM-RT- and CyMol-collected nasal swab specimens from 73 symptomatic students attending a campus health clinic were positive for a respiratory virus in 56.2% of subjects by multiplex PCR testing, including influenza A and B viruses, rhinovirus/enteroviruses, coronaviruses, respiratory syncytial virus, parainfluenza viruses, metapneumovirus, and adenovirus. Detection by PCR was equivalent in UTM-RT- and CyMol-collected specimens and in self- and staff-collected swabs. Direct fluorescent antibody (DFA) testing was substantially less sensitive (23.3%) than multiplex PCR, and DFA testing from UTM-RT-collected swabs was more sensitive than that from CyMol-collected swabs. These data indicate that an alcohol-based transport medium such as CyMol preserves respiratory virus integrity, rapidly inactivates viruses, and is compatible with PCR-based respiratory diagnostics.

The clinical and public health importance of influenza and other respiratory viruses has greatly accelerated the development and optimization of highly sensitive molecular diagnostic tests, but evaluations of key preanalytical components, such as swabs and transport media, remain very limited.

Improvements in the design of collection swabs, such as the Copan FLOQSwab, increase the yield and quality of sample collection for the diagnosis of respiratory virus infections (3, 16). However, few published studies have evaluated viral transport media for molecular-based assays (13). The implementation of a collection and transport system that could inactivate influenza A or other respiratory viruses on collection, preserve cell morphology and viral antigens for rapid testing, and stabilize the viral nucleic acid (NA) for molecular testing would assist in the public health monitoring of respiratory outbreaks as well as in individual patient diagnosis. Several virus inactivation methods have been reported, but few are practical for use during sample collection (4, 14). During a pandemic, when the transport of samples from various collection sites to central laboratories is required and when the large number of samples quickly overwhelms laboratory staff, virus inactivation at collection would reduce the biohazard risk from sample leakage during transit and sample processing, potentially simplify transportation requirements, and ensure specimen integrity.

In this study, we evaluated CyMol, a new alcohol-based transport medium. Specifically, we assessed (i) the stability over time of influenza A nucleic acid quantitation in CyMol as a measure of specimen integrity, (ii) the loss of viability of virus in CyMol transport media as a measure of biosafety, and (iii) the compatibility of CyMol with antigen- or nucleic acid-based diagnostic tests for respiratory tract viruses.

MATERIALS AND METHODS

Mock-infected samples of flocked nasal midturbinate swabs (Copan Italia SpA, Brecia, Italy) collected in CyMol transport medium (Copan) were compared to flocked nasal swabs collected in universal transport medium (UTM-RT; Copan) and to a dry flocked swab collection. Briefly, mock-infected samples consisted of 50 μl of influenza A viral lysate (H3N2; A/Victoria/3/75; approximately 3.0×10^6 genome equivalents [ge]) diluted in an influenza A-negative nasopharyngeal swab (NP) sample matrix adsorbed onto duplicate flocked nasal swabs and inserted into a 1-ml CyMol or UTM-RT sample collection tube or maintained as a dry swab in a transport tube.

The stability and recovery of influenza A viral RNA (H3N2) was assessed for each collection system during a 21-day period at four different temperatures, −20°C, 4°C, room temperature (RT; approximately 22°C), and 37°C, to simulate transport under temperate, cooler, or tropical conditions. The collection tubes with swabs were held at each temperature for 1, 7, 14, or 21 days before nucleic acid extraction. To release the virus from the swab, the CyMol and UTM-RT mock-infected samples were briefly vortexed, and the swab then was discarded.

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TABLE 1. Influenza A virus quantitation by temperature, days of storage, and transport media

Collection system and storage temp[b] (°C)	Quantitation (log_{10}) of influenza A virus on day[a]:			
	1	7	14	21
UTM-RT				
−20	5.42	5.37	5.33	5.35
4	5.33	5.40	5.36	5.32
22	5.31	5.29	5.27	**4.08**
37	5.23	4.71	**4.03**	**3.08**
CyMol				
−20	5.28	5.37	5.16	5.41
4	5.26	5.32	5.36	5.39
22	5.07	5.04	4.86	**4.63**
37	4.71	**4.36**	**3.29**	**1.46**
Dry				
−20	5.22	4.99	5.12	5.03
4	5.00	**4.35**	5.10	4.98
22	4.98	**4.30**	**4.64**	**3.45**
37	4.74	**4.01**	**3.59**	**1.05**

[a] Quantitative RT-PCR targeting the influenza A matrix gene was used to assess viral genomic copy numbers in duplicate, and results are expressed as log_{10} genome equivalents. Significant values ($P < 0.05$) are indicated in boldface (comparison with mean values for day 1 at −20°C; SPSS version 18).

[b] Room temperature was 22°C.

For the dry swab collection, 1 ml of UTM-RT was added after mock storage, and the swab was vortexed and discarded.

To recover viral RNA, a 500-μl aliquot of the mock-infected specimens was extracted by easyMAG (bioMérieux, Montreal, Canada) and eluted in 60 μl. Five μl of purified NA was tested by quantitative matrix influenza A reverse transcription-PCR on a Roche LightCycler 2.0. The influenza A CDC real-time reverse transcription-PCR assay was carried out in a 20-μl final reaction volume using the QuantiTect probe reverse transcription-PCR kit (Qiagen, Mississauga, Canada) and final primer and probe concentrations of 0.8 and 0.2 μM, respectively (19).

Inactivation of influenza A virus at RT was measured at baseline and after 5-, 10-, 20-, and 30-min exposures to UTM-RT and CyMol collection media with two influenza A subtypes: H3N2 (A/Victoria/3/75) and pandemic H1N1 (A/California/04/09-like, H1N1 patient isolate). Virus viability was assessed by duplicate inoculation into R-mix shell vial culture (Diagnostic Hybrids Inc., Athens, OH) at a 1:10 dilution, followed by immunofluorescent staining after 48 h of incubation at 37°C. The effectiveness of inactivation at RT after a 30-min exposure to CyMol was tested on five additional influenza A subtypes (H1N1, H6N5, H8N4, H10N8, and H15N8) with UTM-RT as the comparator. Virus inactivation was not assessed for the dry swab collection.

For clinical validation, 73 university students attending the Campus Health Centre at McMaster University for symptomatic upper respiratory tract infections were invited to collect nasal flocked midturbinate swabs for respiratory virus diagnosis by a commercial multiplex PCR, the xTAG RVP (Luminex Molecular Diagnostics, Austin, TX). Students were enrolled between January and April 2009. One self-collected and one staff-collected swab, from opposite nostrils, were collected. Swabs were placed, in computer-randomized order, into either CyMol or UTM-RT and transported to the research laboratory. All nasal swabs were extracted with easyMAG and tested by multiplex PCR (respiratory virus panel version 1.0 [RVP]; Luminex, Austin, TX). The RVP xTAG assay detects 16 different types and subtypes of common respiratory viruses, including pandemic influenza A H1N1 (11, 12). All swabs were assayed by direct fluorescent antibody (DFA). Briefly, the samples were vortexed and spun, and a 10-well slide was prepared from the phosphate-buffered saline (PBS)-resuspended cell pellet followed by cold acetone fixation and staining with fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies for influenza A and B viruses, respiratory syncytial virus (RSV), parainfluenza virus 1, 2, and 3, metapneumovirus, and adenovirus (Diagnostic Hybrids Inc., Athens, OH) according to the manufacturer's instructions. The study was approved by the McMaster University Research Ethics Board, and all subjects gave written, informed consent.

Statistical testing was performed in SPSS (for Windows; version 18) using means of log-transformed viral copy numbers, generalized linear models for the comparison of media, temperatures over time (main-effect model without interactions), and McNemar's test for paired comparisons. $P < 0.05$ was interpreted as statistically significant. Agreement was assessed as raw agreement and agreement beyond chance (kappa) with 95% confidence intervals (CI).

RESULTS

Specimen integrity, as measured by the quantification of influenza A virus RNA in mock-infected specimens, varied by temperature, duration of storage, and type of transport medium (Table 1 and Fig. 1). Temperature and duration of storage had marked effects on specimen integrity, with 1- to 2-log decreases in viral quantity at 37°C at 14 days in all three transport media ($P < 0.001$) and up to 5-log decreases by 21 days. Effects were less marked at room temperature, and viral quantitation was stable for up to 21 days at −20 or 4°C in all three collection systems.

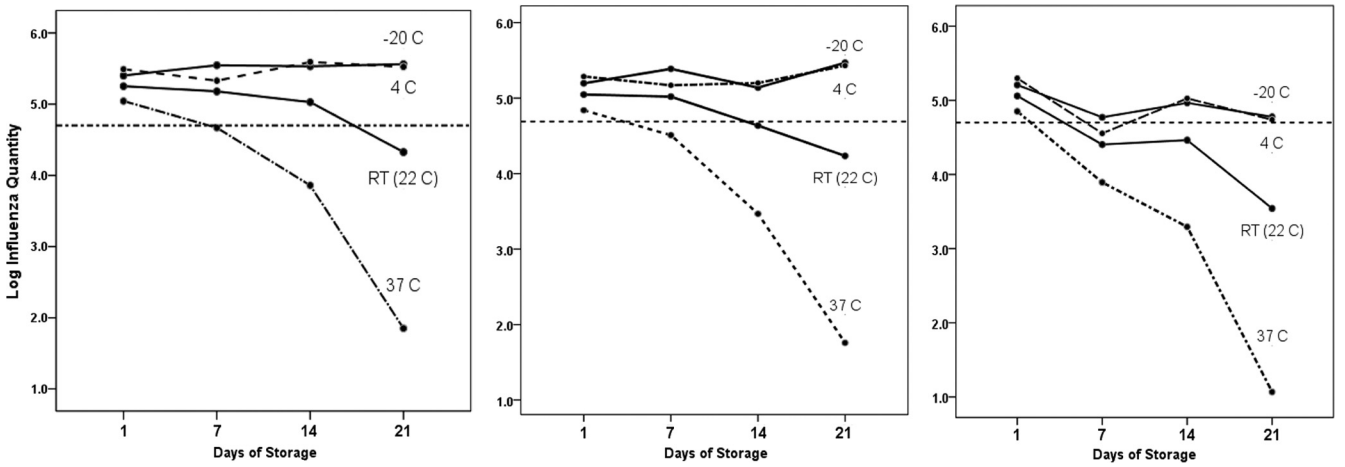

FIG. 1. Modeled effects of temperature and days of storage on influenza A quantitation for three collection methods: universal transport medium from Copan Italia SpA (UTM-RT; left), CyMol from Copan Italia (center), or dry swab without transport medium (right). The horizontal line at 4.7 indicates a 0.5-log difference from baseline values. Statistics were calculated using a main-effect generalized linear model (SPSS version 18.0).

TABLE 2. Comparison of PCR and DFA positivity rates for CyMol to those for UTM-RT and for self-collected nasal swabs to those for staff-collected nasal swabs

Test result[b]	Positivity rate (%, no./total no.) by medium (n = 73 students)			P^a	Positivity rate (%, no./total no.) by collection method		P^a
	Total	CyMol	UTM-RT		Self collected	Staff collected	
PCR							
Positive	56.3 (41/73)	46.6 (34/73)	49.3 (36/73)	0.77	45.2 (33/73)	50.7 (37/73)	0.25
Negative	43.8 (32/73)	53.4 (39/73)	50.7 (37/73)		54.8 (40/73)	49.3 (36/73)	
DFA							
Positive	23.3 (17/73)	11.0 (8/73)	21.9 (16/73)	0.01	13.7 (10/73)	19.2 (14/73)	0.34
Negative	76.7 (56/73)	87.7 (64/73)	76.7 (56/73)		84.9 (62/73)	79.5 (58/73)	

[a] P value for paired comparisons by McNemar test (SPSS version 18).
[b] For the comparison of PCR to DFA, $P < 0.001$.

The type of collection system had less effect than temperature or duration of storage. Both CyMol and UTM-RT collection systems were essentially identical and were superior to dry swab collection. Equivalent viral quantification (within 0.5-log copies from baseline values) was found in CyMol and UTM-RT at −20, 4, and 22°C up to 14 days. At 21 days, CyMol-collected swabs had a higher viral copy number than UTM-RT-collected swabs (4.63 versus 4.08 log copies), but there was no overall difference between CyMol and UTM-RT in a statistical model adjusted for temperature and days of storage (mean difference, 0.21 log copies; $P = 0.13$). In contrast, dry swabs were associated with lower viral quantities at 22 and 37°C at all time points beyond 1 day. However, dry swab collection yielded stable quantification at 4 and −20°C for up to 21 days.

To assess the inactivation of influenza virus in CyMol transport medium, mock-infected specimens stored for various times at RT were tested. After RT exposure to the CyMol transport medium at baseline and for 5, 10, 20, and 30 min, influenza A H3N2 and pandemic H1N1 mock-infected samples were inactivated and unable to grow in shell vial culture. The exposure of five other influenza A virus subtypes (H1N1, H6N5, H8N4, H10N8, and H15N8) to CyMol for 30 min also resulted in the complete inactivation of virus infectivity. In contrast, UTM-RT maintained influenza A infectivity for all seven strains at RT for all time points tested.

Of the 146 clinical samples collected from 73 students with an upper respiratory tract infection, 41 (56.2%) were positive for at least one respiratory virus by the RVP assay, and 17 (23.3%; $P < 0.001$) were positive by DFA. Of 41 students with a respiratory virus detected by PCR, 9 had influenza A (6 H1 and 3 H3), 8 had influenza B, 5 had enterovirus or rhinovirus (the RVP assay does not distinguish between these), 15 coronaviruses (8 229E, 3 NL63, and 4 OC43), 2 metapneumovirus, and 1 each of adenovirus and RSV. Twenty-nine of these were positive for the same virus in both CyMol and UTM-RT, 5 were positive only in CyMol (2 in the first swab, 3 in the second), 7 were positive in UTM-RT (2 in the first swab, 5 in the second), and 32 were negative in both swabs and transport media. The overall positivity was similar for UTM-RT and CyMol (Table 2): swabs taken in UTM-RT were positive in 36/41 cases (87.8%), while those taken in CyMol were positive in 34/41 cases (82.9%; $P = 0.77$ by the McNemar test for paired comparison). For the comparison of the two transport media, raw agreement was 61/73 (83.6%) and kappa (agreement beyond chance) was 0.67 (95% CI, 0.50 to 0.84).

Comparison of self-collected (first swab) and staff-collected (second swab) samples revealed good agreement based on the PCR results. Of 41 RVP-positive students, 33 (80.5%) were positive in self-collected swabs and 37 (90.2%) were positive in staff-collected swabs ($P = 0.25$ by McNemar test). For the comparison of self- and staff-collected swabs, raw agreement was 61/73 (83.6%) and kappa was 0.67 (95% CI, 0.50 to 0.84).

Nasal swabs tested by DFA detected 17 of 73 (23.3%) students with a viral infection: 6 influenza A virus, 8 influenza B virus, and 3 metapneumovirus. However, 33 samples (22.6%) had an insufficient cell quantity (<25 cells/smear) for DFA. Fifteen (88.2%) of the 17 DFA positives also were positive by RVP for the same virus. Two DFA metapneumovirus positives were not confirmed by either RVP or by a metapneumovirus-specific PCR: one was positive only for enterovirus/rhinovirus, and the other was negative by PCR. RVP testing detected an additional six positive students with viruses included in the DFA panel: three influenza A virus and one each of RSV, MPV, and adenovirus. RVP testing also detected an additional 19 virus-positive subjects for viruses that were not included in the DFA panel (15 coronavirus and 5 rhinovirus/enterovirus, including one dual infection), for a total of 41 subjects positive for respiratory viruses detected by RVP and 17 subjects positive by DFA ($P < 0.001$).

Swabs taken in UTM-RT were DFA positive in 16/17 subjects (94.1%), whereas CyMol yielded 8/17 subjects (47.1%, $P = 0.01$). Raw agreement was 63/73 (86.3%), and kappa was 0.51 (95% CI, 0.26 to 0.76). Insufficient cell counts were less common with UTM-RT (7/73 [9.6%]) than with CyMol (26/73 [35.6%, $P < 0.001$]). Eight DFA or RVP discordant sample pairs were assayed for beta-actin as a measure of sample adequacy, and greater quantities of beta-actin were obtained in the UTM-RT than the CyMol collection system (log 3.97 and 2.77, respectively; mean difference, 1.20; 95% CI, 0.35 to 2.05; $P = 0.01$). Staff collection detected 14/17 (82.4%), whereas self collection detected 10/17 (58.8%; $P = 0.34$ by McNemar test). Raw agreement was 63/73 (86.3%), and kappa was 0.50 (95% CI, 0.24 to 0.77).

DISCUSSION

Molecular-based assays are used widely for the diagnosis of viral respiratory infections of clinical or public health importance. However, these require collection and transportation to centralized laboratories, and they may be influenced by poorly understood aspects of collection and transportation. During a pandemic, key preanalytical issues include rapid and stable transport and storage of specimens (preferably at ambient temperature), biosafety of specimens in case of leakage or inappropriate manipulation, and timely processing.

In this study, these preanalytical issues were addressed with three sample collection systems that have potential utility in an influenza pandemic. We assessed mock-infected and clinical nasal flocked swab specimens placed into CyMol or UTM-RT medium or kept as a dry swab. We found that CyMol, an alcohol-based transport medium, enabled the reliable quantitation of virus for at least 14 days at room temperature, rapidly inactivated influenza viruses, and was equivalent to UTM-RT for the multiplex PCR detection of influenza and other respiratory viruses in flocked nasal midturbinate swabs.

CyMol is an alcohol-based medium for the collection and preservation of cells that is compatible with morphological studies of cells (cytology) and with molecular-based assays. Alcohol has been shown previously to preserve viral RNA and DNA for PCR analysis of respiratory viruses. Krafft et al. evaluated ethanol-fixed nasal swab specimens as a surveillance strategy for influenza and adenovirus testing (10). They found that storage in 100% ethanol at 15 to 35°C preserved viral RNA and DNA suitable for detection by real-time PCR for up to 6 months, but viral RNA integrity was variable. The amplification of template sizes larger than 200 bp was unreliable and potentially limits the usefulness of ethanol-preserved samples for molecular subtyping and genetic characterization (5, 17).

In our study, the ability of CyMol and UTM-RT to reliably preserve influenza A viral RNA over time under various storage conditions was investigated using a quantitative influenza A virus matrix PCR. The amplification of nucleic acid targets of up to 400 bp was demonstrated by the multiplex PCR testing of CyMol-collected nasal specimens. Importantly, CyMol preserved influenza virus for at least 21 days at ambient or cooler temperatures, which may greatly facilitate transportation for clinical, public health, and research purposes. This may be particularly important for the collection of specimens in sites remote from the referral laboratory, including isolated communities. Viral nucleic acid quantities deteriorated more rapidly at higher temperatures.

UTM-RT is widely used as a viral transport medium, but little has been published regarding the stability of respiratory viruses at various temperatures and storage times. We found that samples in UTM-RT were stable at room temperature for up to 14 days and were stable for at least 21 days if refrigerated or frozen. Additionally, UTM-RT has been reported to be superior to other transport media for room-temperature storage and shipping in terms of the preservation of viability (7, 16).

The stable recovery of influenza A viral RNA has been demonstrated previously with viral transport media stored at −20 and −80°C over time (16, 18). RNA recovery declined after four freeze-thaw cycles, but viral cDNA remained stable even after multiple freeze-thaws.

Stabilizing reagents, such as RNAlater (Ambion) and AVL buffer (Qiagen), may reduce viral RNA degradation and maximize recovery. After RT storage for 20 h, Forster et al. found no loss in viral RNA recovery in the presence of RNAlater (6). Blow et al. found that AVL-stabilized samples showed no RNA degradation for up to 35 days at −20 and 4°C, but the degradation of viral RNA was noted after 7 days at RT and 2 days at 32°C (2).

During a pandemic or in resource-poor settings, it may be necessary to use dry swab collection systems when other transport media are unavailable. In anticipation of such needs, we evaluated dry swabs and found them to be inferior to CyMol or to UTM-RT at ambient temperature or at 37°C, with measurable deterioration within 1 to 7 days. However, if refrigerated or frozen, influenza virus quantification was stable for up to 21 days in dry flocked swabs without any transport medium. The stability at room temperature differs from that reported by Moore et al., who recovered viral RNA for up to 15 days at RT by nucleic acid sequence-based amplification (NASBA) (13). Our methods for quantification by PCR may be more precise, and our findings indicate a relative, not an absolute, decrease in viral quantification after 1 day. An alcohol-based collection medium such as CyMol has the added advantage of rapidly rendering specimens noninfectious, reducing the risk to couriers and laboratory staff from sample leakage or laboratory manipulation. Virus inactivation at the source may reduce packaging and transportation costs if a lower biohazard level can be assigned to such diagnostic specimens in the future. Consequently, CyMol is a potential alternative for safe sample collection and transportation during influenza or other respiratory virus pandemics.

A variety of solvents and detergents have been investigated for their ability to inactivate viral infectivity (4, 14). Alcohol is widely used as a disinfectant to inactivate viruses and bacteria, and its effectiveness on enveloped viruses has been well documented (8, 9, 15). The inactivation of infectivity also occurs with the RNA-stabilizing agent AVL, although RNAlater maintains infectivity (1, 2). We showed that CyMol rapidly inactivated various influenza viruses without affecting PCR detection and quantification.

Our clinical evaluation involved a blinded, randomized comparison of CyMol to UTM-RT. The two transport systems proved equivalent for PCR testing, giving similar yields. Our study also provides further validation for the self collection of nasal midturbinate flocked swabs as equivalent to staff-collected nasal swabs.

DFA testing was clearly inferior to PCR testing. PCR had a higher tolerance for specimen inadequacy than DFA. In addition, with multiplex PCR testing, we were able to detect coronaviruses and rhinoviruses/enteroviruses, for which DFA testing is not available. Furthermore, and contrary to our expectations, UTM-RT-based media were more sensitive than CyMol-based media for DFA. A greater number of swabs in CyMol had insufficient cell quantity despite randomization to ensure an equal distribution of swabs from the first and second collections swabs. Lower cell counts, coupled with lower beta-actin quantitation, suggest that fewer cells were released from the swab. Lower cell counts also could be due to a lack of

protein in CyMol, which may affect adherence to the glass slides. In contrast, experience with CyMol for cytologic studies did not find any degradation of cells (S. Castriciano, unpublished data). Further work is needed to optimize alcohol-based transport media for antigen detection. For clinical diagnosis or for epidemiologic research, the use of self-collected nasal swabs needs to be coupled with highly sensitive multiplex PCR to achieve high diagnostic yield.

We acknowledge a number of limitations of our study. Virus stability and infectivity data were derived from mock infections and may not represent the full range of diagnostic situations in which virus collection takes place. For the clinical validation, there were insufficient positive samples for a number of respiratory viruses, limiting our ability to draw specific conclusions on the stability of each virus in CyMol. We used one extraction method (easyMAG) and cannot generalize our findings to all extraction systems. Importantly, while CyMol will enable the safer collection and transportation of specimens, the inability to culture inactivated virus may be a limitation for reference laboratories charged with such requirements for antigenic characterization or for phenotypic drug resistance testing. The majority of routine respiratory testing currently is done either by antigen detection or by molecular methods, and the need for routine culture is likely to be greatly reduced in the future.

We conclude that the alcohol-based transport medium CyMol enabled the preservation of virus for prolonged periods of time at ambient temperatures while eliminating potential biohazard to courier and laboratory personnel. CyMol enabled the safe self collection of specimens with high yield when coupled with multiplex PCR, and this technique may facilitate earlier diagnosis of respiratory virus infections in clinical and epidemiologic settings.

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